

PAPER • OPEN ACCESS

Biological activity of chitosan aspartate and its effect on germination of test seeds

To cite this article: Z Khaptsev *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **723** 022074

View the [article online](#) for updates and enhancements.



The Electrochemical Society
Advancing solid state & electrochemical science & technology
2021 Virtual Education

Fundamentals of Electrochemistry:
Basic Theory and Kinetic Methods
Instructed by: **Dr. James Noël**
Sun, Sept 19 & Mon, Sept 20 at 12h–15h ET

Register early and save!



Biological activity of chitosan aspartate and its effect on germination of test seeds

Z Khaptsev¹, T Lugovitskaya², A Shipovskaya³ and K Shipenok³

¹Saratov State Vavilov Agrarian University, 335, Sokolovaya St., Saratov, 410005, Russia

²Ural Federal University, 19, Mira St., Ekaterinburg, 620002, Russia

³Saratov State University, 83, Astrakhanskaya St., Saratov, 410012, Russia

E-mail: dfst@list.ru

Abstract. A polymer salt, chitosan aspartate, was obtained by dissolving chitosan in an aqueous solution of L-aspartic acid. It was found that polysalt macromolecules in an aqueous medium exhibit the properties of a cationic polyelectrolyte with a partially compensated positive charge. Chitosan aspartate shows high antibacterial activity and no cytotoxicity. The positive effect of polysalt on germination of watercress test seeds was revealed, which allows us to consider chitosan aspartate as a promising biostimulator of plant growth.

1. Introduction

Currently, an important direction in improving the technology of growing crops is the development of an effective system for the use of plant growth regulators. At the same time, a modern solution is the use of biologically active substances with prolonged action. Plant growth stimulants based on such biologically active substances have a complex effect on the physiological and biochemical processes occurring in the plants. They are non-toxic and safe for humans and the environment due to their origin. The creation of new prolonged action stimulants based on high-molecular biologically active substances requires a comparative study of them for rational use, since this direction is an economically profitable and eco-friendly agricultural practice.

Chitosan (CTS), a deacetylated derivative of the natural polysaccharide chitin, exhibiting a wide range of biologically useful properties, is considered to be one of the promising chemical compounds for creating prolonged action biostimulants. Preparations containing CTS in their composition have a fairly wide potential for use in various fields of agriculture, in particular, in crop production [1-2]. Let us dwell in more detail on some of the biological effects of CTS and its application.

Chitosan activity against phytopathogens. For the first time, the activity of chitosan against a number of plant pathogens has been reported in 1979 by Allan and Hadwiger [3]. The fungicidal effect of this polymer on fungi with different cell wall compositions was found. In later studies, it has been shown that CTS possesses a broad spectrum of fungicidal activity against several phytopathogenic fungi and effectively inhibits their growth at different stages of the life cycle. For example, chitosan is very effective against the *Colletotrichum capsici* fungus, which causes anthracnose in bell pepper (*Capsicum annuum* L.) [4]. Spraying *Pinus sylvestris* L. with CTS solutions results in effective protection of seedlings from scutiger [5]. An effective inhibitory activity of CTS against spores of *Fusarium eumartii*, a fungal pathogen of potatoes and tomatoes, has been noted in [6].



Along with the antifungal and antibacterial action, CTS also exhibits antiviral activity. It has been revealed that CTS induces resistance to such a systemic pathogen as bean soft mosaic virus [7]. CTS inhibits by 50–90% the appearance of localized necrotic lesions in tobacco caused by the systemic tobacco mosaic virus [8]. The insecticidal effect of CTS against insect pests affecting agricultural crops has been reported. Thus, treatment of plants with CTS inhibits the growth and reduces the viability of larvae of the oleander aphid *Aphis nerii* and caterpillars of the cotton bollworm *Spodoptera littoralis* [9]. The high efficiency of CTS is manifested against the nodular nematode *Meloidogyne javanica* *in vitro* and *in vivo* under greenhouse conditions [10].

Growth stimulating effect of CTS. Chitosan-containing foliar feeding leads to an increase in fruit weight and an increase in tomato yield [11], an increase in the stem height, green mass, size and number of fruits of *Hibiscus esculentus* L. [12]. Root feeding with this polysaccharide significantly increases the height, canopy diameter, and leaf area of *Capsicum annuum* L. [13]. The dressing of wheat seeds and spraying of a grain crop in large-scale field experiments leads not only to an increase in the vegetation, but also the grains on the ear [14]. Spraying the leaves of coffee plants with CTS increases their vegetative growth [15], while spraying strawberries *Fragaria chiloensis* L. increases the yield [16]. Immersion of grapevine cuttings in a CTS solution promotes rooting and an increase in internodes [17]. An improvement in growth and flowering plant species *Dendrobium formosum* orchids (seed treatment) [18], gladioli *Gladiolus communis* L. (processing of corms and flowers) [19], freesia *Gompey* varieties (processing of corms) [20] have been reported.

Chitosan-containing preparations are also used to create a protective coating on fruits (apples, citrus fruits, kiwi, peaches, pears, cherries), berries (strawberries) and vegetables (cucumbers, carrots) in order to increase their shelf life [16; 21]. The protective barrier formed during the treatment of CTS reduces the loss of water and nutrients, inhibits gas exchange, and prevents the microorganisms growth on the fruits surface.

The use of chitosan-containing nanoparticles in agriculture. In recent years, various methods for the synthesis of CTS nanoparticles and approaches to their use as delivery systems for pesticides, fertilizers, and microelements have been proposed [22, 23]. At the same time, the most widespread are core-shell type nanocomposites [24]. The encapsulation of a chemical in chitosan nanoparticles makes it possible to solve the problems of solubility of plant growth regulators and other agrochemicals. For example, the encapsulation of water-insoluble rotenone in nanomicelles, consisting of an amphiphilic derivative of CTS, significantly increases the solubility of this insecticide [25]. Nanocomposites are also very effective for combating various pests and diseases of agricultural crops. Thus, CTS and sodium tripolyphosphate nanoparticles inhibit the development of the rice fungus *Pyricularia grisea* [26]. In addition, the use of nanoparticles makes it possible to reduce the amount of applied agrochemicals without compromising their effectiveness.

Thus, we can conclude that chitosan-containing preparations (powders, solutions, nanoparticles) have a multitarget activity against phytopathogens and a stimulating effect against a number of plant crops. Therefore, expanding the range of research in this direction, in particular, testing of chitosan aspartate, a bifunctional derivative of chitosan and L-aspartic acid, is very relevant and has practical significance.

The aim of this work is to obtain and evaluate the viscosity properties of chitosan aspartate, to study its cytotoxicity and antibacterial activity, as well as to study the germination of test seeds of radish *Raphanus sativus* and watercress *Lepidium sativum* in the presence of chitosan aspartate.

Note that the choice of L-aspartic acid (AspA) for the preparation of the polymer salt is due to its special biological properties. This amino acid is present in plant proteins. It is the first synthesis product in symbiotic nodule bacteria. It performs important functions in the exchange of nitrogen-containing substances, in particular, it is formed in the roots of plants after the introduction of nitrogen fertilization, it utilizes ammonia with the transformation into asparagine, which is necessary for plants to synthesize amino acids, amides and proteins.

2. Materials and methods

An industrial sample of CTS with a molecular weight of 200 kDa, a deacetylation degree of 82 mol% (Bioprogress, Russia) and AspA of analytical grade (Bioamid, Russia) were used. Stock solutions with specified concentrations of CTS (C_{CTS}) and AspA (C_{AspA}) were prepared by mixing weighed portions of the polymer and acid in distilled water at 50 °C. Working solutions were obtained by diluting the stock solution with distilled water. The concentration of the working solutions depended of the research method. The prepared solutions were filtered through a Schott filter No. 160.

The viscosity properties of solutions were evaluated in an Ubbelohde capillary viscometer with a capillary diameter of 0.56 mm at 25 °C. The time of the outflow of the polymer and acid solutions was determined, according to which the viscosity number ($\eta_{\text{sp}}/C_{\text{CTS}}$, dL/g) was calculated according to the standard method.

Cytotoxicity was investigated *in vitro* using the MA-104 rhesus monkey embryo kidney epithelial cell line model. In flasks with a nutrient medium DMEM supplemented with 10% FBS (Hyclone) and 1% antibiotics (Sigma), a solution of chitosan aspartate and a suspension of daily culture of MA-104 cells (110 thousand cells per 1 mL) were added. The cells were cultured for 3 days in a CO₂ incubator at a constant temperature (37 °C), humidity (90%), and CO₂ content (5%). Cell spreading and proliferation were observed using an inverted microscope Biolam P-3 (LOMO, Russia). The culture medium in the vials was not changed until the end of the observation period.

The antibacterial activity was determined by diffusion in agar with a daily culture of the reference *Staphylococcus aureus* 209 P strain. Sterile Petri dishes with a diameter of 100 mm agar medium for the cultivation of microorganisms were used. Aliquots of a solution of chitosan aspartate of various concentrations were added to the wells of the agar medium, kept for 1 h at room temperature for diffusion of the drug into agar, thermostated at 37 °C for 18–20 h, and the diameter of the growth inhibition zone of the test strain was measured (D , mm).

In a separate series of experiments, the effect of the chitosan aspartate solution on seed germination was evaluated. The test seeds of *Raphanus sativus* and *Lepidium sativum* were used. The experiment to assess the germination of test seeds was carried out in glass Petri dishes with closed lids for 35 hours. A gauze cloth moistened with 10 mL of CTS solution in AspA or 10 mL of distilled water (control) was placed on the bottom of the Petri dish. Three parallel experiments were carried out using 50 pcs. test seeds in each. Germination was assessed by the ratio of the number of germinated test seeds to the total number of seeds taken into the experiment and expressed as a percentage. The choice of this indicator is due to the fact that the survival and productivity of a plant organism is largely determined by the characteristics of seed germination. When counting germinated seeds, only normally germinated seeds were taken into account. Swollen, but abnormally germinated seeds were considered as non-germinating ones.

3. Results and discussion

Viscosity properties of chitosan aspartate.

During the dissolution of chitosan in an aqueous solution of AspA, the protonation of the polymer amino groups and the formation of a rapidly dissociating polycationic salt, chitosan aspartate (figure 1) [27-28]. Since the viscosity characteristics of the solution used for pre-sowing seed treatment and spraying the green mass of the plant determine the penetrating ability of the biologically active substance into the plant organism and its duration of action, at the first stage, the hydrodynamic properties of an aqueous solution of polymer salt were studied.

A study of the hydrodynamic properties of aqueous solutions of chitosan in Asp showed that the chitosan aspartate macromolecules exhibit the properties of a polyelectrolyte with a partially compensated charge: the viscosity number $\eta_{\text{sp}}/C_{\text{CTS}}$ increases with decreasing C_{CTS} , while the dependence $\eta_{\text{sp}}/C_{\text{CTS}} = f(C_{\text{CTS}})$ passes through a maximum and has a dropping branch (figure 2). It is likely that the presence of free Asp ions causes some screening of the polycation charge. In addition, polymeric salt macromolecules exhibit mixed polyelectrolyte/ionomer behavior when only part of the low molecular weight counterions are in a “bound” state with the macroion.

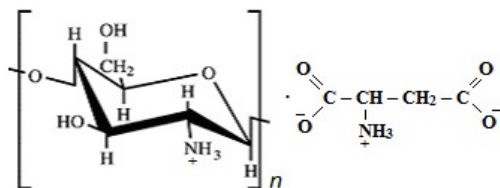


Figure 1. Chitosan aspartate structure.

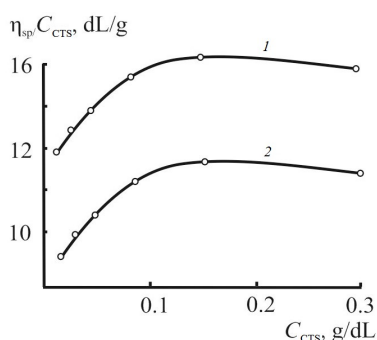


Figure 2. Concentration dependence of the viscosity number of aqueous chitosan solutions in Asp with $C_{AspA} = 0.4$ (1) and 0.8 g/dL (2), 25°C .

Thus, chitosan aspartate in an aqueous medium exhibits the properties of a cationic polyelectrolyte. The presence of an effective positive charge on the macromolecule determines the binding of the polymer with the anionic components of the surface structures of cells and microorganisms due to electrostatic interaction. The viscosity indicators of chitosan aspartate solutions, along with its high molecular weight, determine the possibility of prolonged action of drugs based on it.

Cytotoxicity and antibacterial activity of chitosan aspartate.

Cytotoxicity and antibacterial activity of aqueous solutions of chitosan aspartate were studied. It was found that the addition of a polymeric salt solution to the nutrient medium positively affects the rate of spreading and proliferation of epithelial cells MA-104. So, after 1 h of cultivation in a medium with additive (chitosan + Asp), there were 2 times more spread cells were observed than in the control. In control, the formation of a mature monolayer of epithelial cells MA-104 was observed only on the 3rd day of cultivation. The results obtained indicate not only the absence of cytotoxic effect of chitosan aspartate, but also a significant improvement in the growth qualities of the nutrient medium with the addition of polysalt, leading to an acceleration of the growth of the MA-104 cell population.

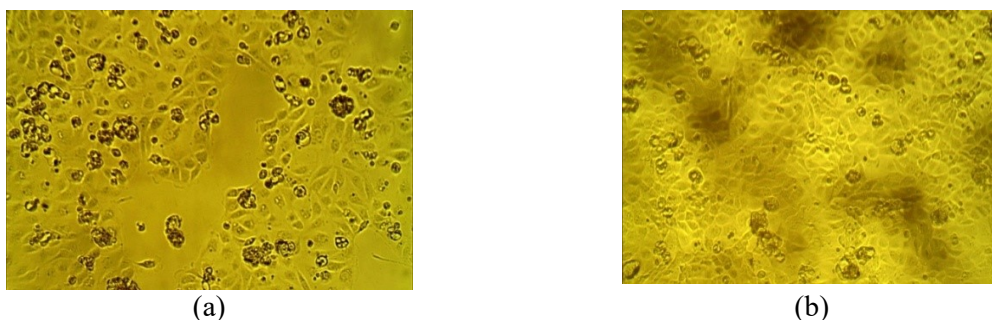


Figure 3. Epithelium-like cells of the MA-104 line after 48 h of cultivation without the addition (a) and with the addition (b) of chitosan aspartate with $C_{CTS} = 0.1$ g/dL, $C_{AspA} = 0.1$ g/dL, dilution 1:10, magnification $100\times$.

It was revealed that chitosan aspartic acid salt exhibits high antibacterial activity against the opportunistic culture of *Staphylococcus aureus*. All studied solutions of this polymeric salt successfully inhibited the growth of colonies of *Staphylococcus aureus* 209 P. At the same time, clear

zones on bacterial lawn were formed, the radius of which decreased as the concentration of chitosan aspartate decreased (table 1).

Table 1. Antibacterial activity of chitosan aspartate.

Concentration of chitosan and AspA in a solution		Diameter of zone of growth inhibition of colonies of <i>S. aureus</i> 209 P
C_{CTS} , g/dL	C_{AspA} , g/dL	D , mm
0.04	0.6	20
0.08		26
0.16		28
0.32		30
0.64		32
1.20		33

The experiments carried out allow us to state that the combination of two biologically active substances (CTS and AspA) in one preparation leads to a synergistic effect and the formation of a salt form of CTS with high biological activity. Presumably, these properties of chitosan aspartate should stimulate cell division and directed regulation of the main growth processes in plants. This circumstance predetermined the formulation of an experiment to assess the effect of chitosan aspartate on the germination of test seeds.

Effect of chitosan aspartate on germination of test seeds

It was found that the germination of test seeds of *Raphanus sativus* in CTS-AspA substrates of various concentrations and in distilled water is practically identical (figure 4a; 4b). Intensive germination of seeds (29–33 pcs.) begins after ~ 22 h of exposure. After ~ 35 h, the number of germinated seeds in all experiments (chitosan-containing and control substrates) is 39–40, and the germination capacity is 78–80% (table 2).

For test seeds of watercress *Lepidium sativum* (figure 4c, 4d), intensive germination (9–18 pcs) in CTS-AspA substrate and H₂O is observed after ~12–13 h of exposure. In this case, the largest number of germinated seeds is realized in chitosan aspartate medium and depends on the concentration of its solution. So, after ~30–35 h of exposure, the number of germinated seeds in a chitosan-containing substrate with $C_{CTS} = 0.075$ g/dL and $C_{AspA} = 0.05$ g/dL reached 42–43 pieces, with $C_{CTS} = 0.038$ g/dL and $C_{AspA} = 0.025$ g/dL – 28 pcs, in distilled water – 26 pcs. Seed germination in the medium with $C_{CTS} = 0.075$ g/dL and $C_{AspA} = 0.05$ g/dL is 86%, $C_{CTS} = 0.038$ g/dL and $C_{AspA} = 0.025$ g/dL – 56%, in distilled water – 52% (table 2).

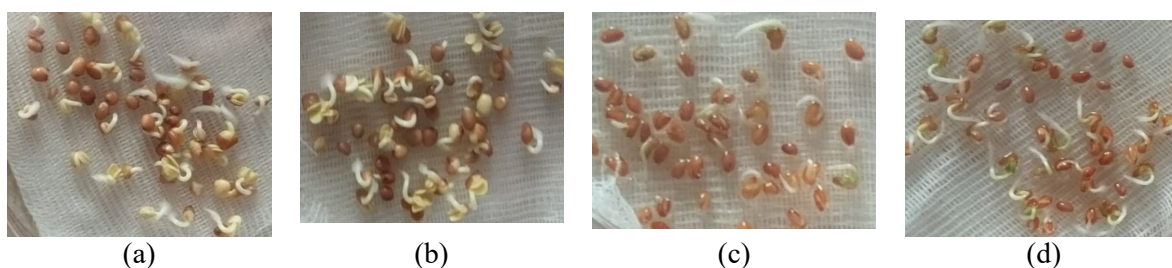


Figure 4. Test seeds of *Raphanus sativus* (a, b) and *Lepidium sativum* (c, d), germinated in distilled water (a, c) and chitosan aspartate solution with $C_{CTS} = 0.038$ g/dL, $C_{AspA} = 0.025$ g/dL (b) and $C_{CTS} = 0.075$ g/dL, $C_{AspA} = 0.050$ g/dL (d).

Thus, when chitosan aspartate was used as a biologically active substance of prolonged action, its positive effect on the germination of watercress test seeds was observed.

Table 2. Germination of test seeds of *Raphanus sativus* and *Lepidium sativum* in distilled water and in chitosan aspartate medium.

Test seeds	C _{CTS} , g/dL	C _{AspA} , g/dL	Number of germinated seeds, pcs							Germination, %
			Time, h							
			12	13	15	22	24	30	35	
<i>Raphanus sativus</i>	—	—	0	3	5	31	36	37	39	78±2
	0.075	0.050	0	2	3	29	31	37	40	80±2
	0.038	0.025	0	2	3	33	33	35	39	78±2
<i>Lepidium sativum</i>	—	—	9	9	11	25	25	25	26	52±2
	0.075	0.050	16	18	21	38	43	42	43	86±2
	0.038	0.025	13	18	23	25	28	28	28	56±2

In conclusion, it can be stated that as a result of the interaction of chitosan with Asp in an aqueous medium, a visually homogeneous molecular solution of the corresponding salt is formed. The biological properties revealed in experiments suggest that chitosan aspartate can be used to obtain biologically active stimulants of prolonged action for green crops. We also believe that chitosan aspartate can be very promising for stimulating molecular defense systems in plants, which is the subject of further research.

References

- [1] Malerba M and Cerana R 2016 *Int. J. Molec. Sci.* **17**(7) 996
- [2] Mujtaba M, Khawar K M, Camara M C, Carvalho L B, Fraceto L F, Morsi R E and Wang D 2020 *Int. J. Biol. Macromol* **154** 683
- [3] Hamel L P and Beaudoin N 2010 *Planta* **232**(4) 787
- [4] Ali A and Noh N M 2015 *Food Pack. Shel. Life* **3** 56
- [5] Aleksandrowicz-Trzcinska M, Bogusiewicz A, Szkop M and Drozdowski S 2015 *Forests* **6** 3165
- [6] Terrile M C, Mansilla A Y, Albertengo L, Rodríguez M S and Anahi Casalongue C 2015 *Pest Manag. Sci* **71** 668
- [7] Kulikov S N, Chirkov S N, Ilina A V, Lopatin S A and Varlamov V P 2006 *Appl. Biochem. Microbiol* **42** 200
- [8] Davydova V N, Nagorskaya V P, Gorbach V I, Kalitnik A A, Reunov A V, Soloveva T F and Ermak I M 2011 *Appl. Biochem. Microb* **47** 103
- [9] Badawy M E I, Ahmed F and El-Aswad A F 2012 *Plant Protect. Sci* **48** 131
- [10] El-Sayed S M and Mahdy M E 2015 *Int. J. Chem. Tech. Res* **59**
- [11] Amerany F El, Meddich A, Wahbi S, Porzel A, Taourirte M, Rhazi M and Hause B 2020 *Int. J. Molec. Sci* **21**(2) 535
- [12] Mondal M M A, Malek M A, Puteh A B, Ismail M R, Ashrafuzzaman M and Naher L 2012 *Aust. J. Crop Sci* **6** 918
- [13] Chookhongkha N, Miyagawa S, Jirakiattikul Y and Photchanachai S 2012 *Int. Conference on Agriculture Technology and Food Sciences (ICATFS'2012), Manila, Philippines* 146
- [14] Wang M, Chen Y, Zhang R, Wang W, Zhao X, Du Y and Yin H 2015 *Field Crops Res* **172** 11
- [15] Van S N, Minh H D and Anh D N 2013 *Biocatal. Agric. Biotechnol* **2** 289
- [16] Saavedra G M, Figueroa N E, Poblete L A, Cherian S and Figuero C R 2016 *Food Chem* **190** 448
- [17] Gornik K, Grzesik M, Romanowska-Duda B and Fruit Ornam J 2008 *Plant Res* **16** 333
- [18] Kananont N, Pichyangkura R, Chanprame S, Chadchawan S and Limpanavech P 2010 *Sci. Hortic* **124**(2) 239
- [19] Ramos-Garcia M, Ortega-Centeno S, Hernandez-Lauzardo A N, Alia-Tejagal I, Bosquez-

- Molina E and Bautista-Banos S 2009 *Sci. Horti* **121(3)** 480
- [20] Salachna P, Zawadzinska A and Ecol J 2014 **15** 97
- [21] Kerch G 2015 *Trends Food Sci. Technol* **46(2)** 159
- [22] Maluin F N and Hussein M Z 2020 *Molecules* **25(7)** 1611
- [23] Quinones J P, Garcia Y C, Curiel H and Covas C P 2010 *Carbohydr* **80** 915
- [24] Vanti G L, Masaphy S, Kurjogi M, Chakrasali S and Nargund V B 2020 *Int. J. Biol. Macromol* **156** 1387
- [25] Lao S B, Zhang Z X, Xu H H and Jiang G B 2010 *Carbohydr. Polym* **82** 1136
- [26] Manikandan A and Sathiyabama M 2016 *Int. J. Biol. Macromol* **84** 58
- [27] Lugovitskaya T N, Zudina I V and Shipovskaya A B 2020 *Rus. J. Appl. Chem* **93(1)** 80
- [28] Lugovitskaya T N and Shipovskaya A B 2017 *Rus. J. Gen. Chem* **87(4)** 782